Estradiol increases proteinuria and angiotensin II type 1 receptor in kidneys of rats receiving L-NAME and angiotensin II

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Prospective, placebo-controlled clinical trials suggest that estrogen may have adverse effects on the vascular system in women. The goal of this study was to determine if 17β-estradiol (E2) would have adverse effects on the renovasculature in a rat model of renal injury characterized by low nitric oxide (NO) and high angiotensin II (AngII). We studied female Wistar rats that were sham-operated (sham), ovariectomized (OVX), or ovariectomized and replaced with E2 (OVX/E2). All rats were maintained on a high salt diet and renovascular injury was caused by treating rats with an inhibitor of NO synthase, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), for 14 days, plus AngII on days 11 through 14. L-NAME/AngII treatment, as compared to placebo, caused proteinuria, glomerular injury, and fibrinoid necrosis of renal arterioles in sham-operated rats. Ovariectomy reduced L-NAME/AngII-induced renal damage, whereas E2 treatment increased L-NAME/AngII-induced damage in OVX rats. In rats treated with L-NAME/AngII, levels of AngII type 1 receptor (AT_1R) protein were higher in the renal cortex of sham and OVX/E2 rats than in OVX rats. AT_1R protein correlated with renal injury. E2 treatment also increased expression of AT_1R mRNA. Thus, under conditions of low NO and high AngII, E2 exacerbated renal injury. E2-mediated increases in renal cortical AT_1R expression may represent a novel mechanism for the adverse renovascular effects of estrogen.

Kidney International (2006) **70,** 1759–1768. doi:10.1038/sj.ki.5001897; published online 4 October 2006

KEYWORDS: estradiol; proteinuria; renal injury; angiotensin II; angiotensin II receptor 1; hypertension

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Received 20 December 2005; revised 4 August 2006; accepted 22 August 2006; published online 4 October 2006

Many observational studies show beneficial cardiovascular effects with hormone replacement therapy.[1–3] However, large-scale, prospective clinical studies suggest that estrogen with and without progestin may not be beneficial and may contribute to vascular pathology. In the Heart and Estrogen/progestin Replacement Study, hormone replacement therapy failed to provide benefit in women with established coronary heart disease.[4] The Women's Health Initiative showed that, compared with placebo, estrogen replacement therapy, alone or with progestin, is associated with an increased risk for vascular disease in postmenopausal women.[5,6] These adverse effects on the vasculature appeared to be widespread as increases in coronary events, stroke, and pulmonary embolism were observed. It has been suggested that these adverse effects of hormone replacement therapy occur in women with underlying cardiovascular disease.[7]

Microalbuminuria is an indicator of renal vascular injury and is a predictor of cardiovascular disease.[8] In Heart and Estrogen/progestin Replacement Study, renal injury was a strong predictor of peripheral arterial disease events.[9] A few studies have examined the effect of estrogen with and without progestin on microalbuminuria in women. One study showed no effect of estrogen plus progestin on microalbuminuria in postmenopausal women with type II diabetes.[10] In contrast, microalbuminuria was increased in premenopausal women using oral contraceptives and in postmenopausal women receiving hormone replacement therapy as compared with women not receiving these medications.[11,12] Oral contraceptive use also increased the risk of macroalbuminuria in women with diabetes.[13] In healthy women and in women with diabetes, oral contraceptive use was associated with an exaggerated renal blood flow response to acute administration of an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker, suggesting that oral contraceptives lead to increased angiotensin II (AngII) activity in the renal circulation.[13]

The goal of this study was to determine if 17β-estradiol (E2) would have adverse effects on the renovasculature in a rodent model of renal injury, and if it did, by what

mechanisms. We chose a model of renal injury that is dependent on low nitric oxide (NO) and on the presence of a well-known mediator of renal injury, AngII. Low NO and high AngII are commonly observed in patients with hypertension.[14] In this animal model of renal injury, rats on a high-sodium diet receive the NO synthase inhibitor N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) for 14 days and AngII for the last 3 days, resulting in the development of proteinuria, glomerular injury, and fibrinoid necrosis of renal arterioles.[15] Using this model, we examined the effects of ovariectomy and E2 replacement on proteinuria, renal histopathology, and renal cortical expression of AngII type 1 receptor (AT_1R) protein and mRNA.

RESULTS

L-NAME/AngII and renal injury

We studied five groups of female Wistar rats: sham-operated rats receiving placebo (control treatment), sham-operated rats receiving L-NAME/AngII, ovariectomized (OVX) rats receiving control treatment, OVX rats receiving L-NAME/AngII, and OVX rats receiving L-NAME/AngII plus E2. All rats received 1% NaCl to drink. Rats were killed at the end of the 14-day treatments without respect to timing of the 4-day estrous cycle and blood was collected for determination of E2 levels. Thus, there was a wide range of E2 levels in the 18 sham-operated rats (range 5–45 pg/ml, mean 16.8 ± 2.6 pg/ml). The mean E2 level in the 18 OVX rats not receiving E2 (8.9 ± 2.3 pg/ml) was significantly lower than that observed in the sham-operated rats ($P < 0.01$ by Student's t-test). E2 levels were significantly higher in OVX rats receiving E2 as compared with OVX rats receiving placebo (Table 1). Consistent with their low-estrogen status, OVX rats had higher body weights, lower uterine weights, and lower uterine/body weight ratios than sham-operated rats and E2-treated OVX rats (Table 1). L-NAME/AngII treatment, as compared to control treatment, increased systolic blood pressure (BP) and this increase in BP was similar in sham-operated OVX and OVX/E2 groups (Figure 1a and Table 1).

In sham-operated rats, proteinuria measured on day 14 was 20-fold higher with L-NAME/AngII treatment as compared with control treatment (Figure 1b and Table 1). This result was not due to differences in urine collections, as 24-h urinary creatinine excretion was similar across groups. Histological evaluation of kidneys from L-NAME/AngII-treated rats revealed glomerular damage and renal vascular injury characterized by perivascular inflammation and

Table 1 | Effect of L-NAME/AngII and E2 treatments on metabolic measurements and histological assessment of renal vascular injury in sham and OVX rats

	Control		L-NAME/AngII		
	Sham (n=8)	OVX (n=8)	Sham (n=10)	OVX (n=10)	OVX/E2 (n=10)
BW (g)					
Day −8	242±7	274±5[a]	256±3	273±4[a]	276±3[a]
Day 14	288±3	335±7[a]	280±5	313±9[a]	260±4[b]
UW					
UW(g)	0.88±0.05	0.22±0.02[a]	0.79±0.06	0.24±0.02[a]	0.91±0.1[b]
UW/BW (mg/g)	30.7±1.5	6.5±0.5[a]	28.4±2.3	7.8±0.5[a]	35.0±4.4[b]
Estradiol (pg/ml)	12.8±2.6	9.9±2.0	20.0±4.1	8±1.7	141±35[a,b,c,d]
Systolic blood pressure (mm Hg)					
Day −1	121±6	117±3	111±4	108±5	103±4
Day 14	122±4	126±5	159±8[c]	153±6[d]	164±9[d]
24-h urine measurements					
Total protein (mg/day)					
Day −1	10.1±0.7	15.7±6.8	10.4±1.9	9.5±1.1	11.0±1.5
Day 14[e]	8.5±0.9	7.0±0.4	167.0±35.3[c]	85.2±34.4[a]	212.4±38.8[b,d]
Creatinine (mg/day)					
Day −1	8.6±1.3	9.4±1.1	9.3±1.3	14.0±1.8	10.3±1.1
Day 14[e]	8.6±1.4	9.8±1.8	10.0±1.6	14.2±5.0	7.7±1.0
Histological assessment of renal damage					
Renal vascular injury score (number of injured vessels per 100 glomeruli)	0±0	0.6±0.3	7.3±1.4[b,c,d]	1.6±0.5[a]	8.1±1.3[b,c,d]
Number of glomeruli (per section)	391±11	372±11	363±15	347±13	369±23

AngII, angiotensin II; BW, body weight; L-NAME, N^{ω}-nitro-L-arginine-methyl-ester; OVX, ovariectomized; OVX-E2, ovariectomized and replaced with E2; UW, uterine weight.
[a]$P < 0.05$ vs sham group receiving same treatment.
[b]$P < 0.05$ vs OVX group receiving L-NAME/AngII treatment.
[c]$P < 0.05$ vs sham group receiving control treatment.
[d]$P < 0.05$ vs OVX group receiving control treatment.
[e]n=6–8 per group.
Data given as mean±s.e.

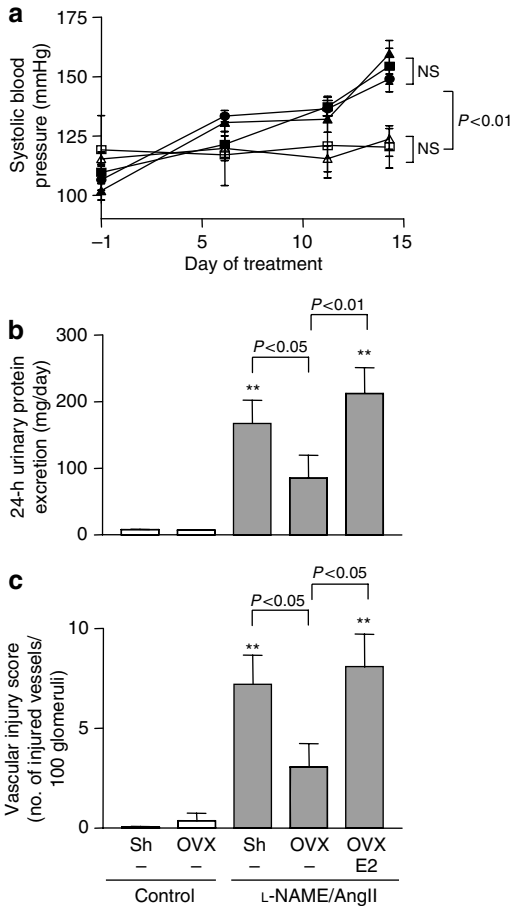

Figure 1 | Effect of estrogen status on systolic BP, proteinuria, and renal vascular injury score in rats receiving L-NAME/AngII or control treatment. Sham-operated (Sh) or OVX female rats drinking 1% NaCl received either the 14-day L-NAME/AngII treatment (gray bars) or control treatment (white bars). Rats were further randomized to receive E2 or placebo starting 1 week before L-NAME/AngII treatment. (**a**) Systolic BP was measured over the course of the study. (**b**) Twenty-four hour urinary protein excretion and (**c**) renal vascular injury score expressed as number of injured vessels per 100 glomeruli were measured at the end of the 14-day treatment. $^{**}P<0.001$ compared with control treatment. Control treatment groups: $n=8$ for BP, $n=7$–8 for urinary protein and renal vascular injury score. L-NAME/AngII treatment groups: $n=10$ for BP, $n=6$–8 for urinary protein, $n=8$–9 for renal vascular injury score. Data given as mean ± s.e.

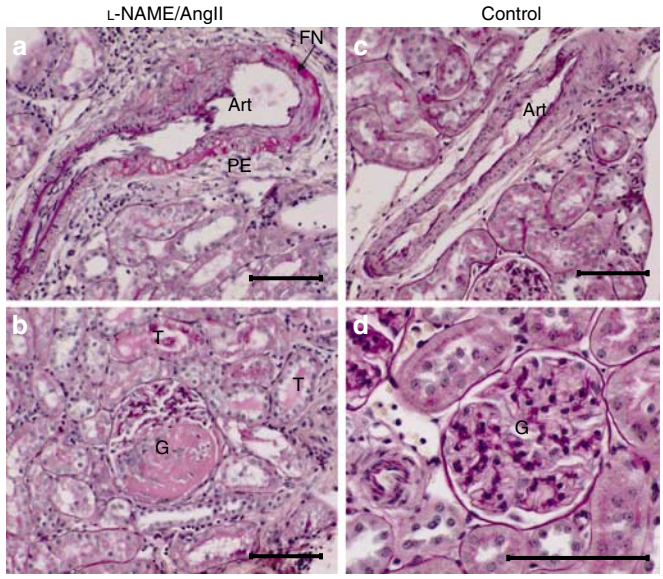

Figure 2 | Renal histopathology of rats receiving L-NAME/AngII and control treatments. Representative sections of (**a** and **b**) damaged kidney from L-NAME/AngII-treated rats and (**c** and **d**) normal kidney from control-treated rats. Right side of renal arteriole (Art) in panel (**a**) shows early, localized fibrinoid necrosis (FN) with destruction of the muscular layer, perivascular edema (PE), and perivascular inflammatory cell infiltrates. Glomerulus (G) in panel (**b**) shows segmental fibrinoid necrosis of the glomerular tuft and mesangiolysis. Tubules (T) in panel (**b**) have debris casts indicating tubular injury. Bar = 100 μm.

fibrinoid necrosis (Figure 2). Scoring of the renal vascular injury revealed significant damage in L-NAME/AngII-treated, sham-operated rats compared with control-treated, sham-operated rats (Table1 and Figure 1c).

Ovariectomy significantly reduced L-NAME/AngII-induced proteinuria and renal vascular injury. The protective effect of ovariectomy was completely reversed by administration of E2 (Figure 1b and c, and Table 1). Compared with OVX rats not receiving E2, OVX rats with E2 replacement had a significant increase in L-NAME/AngII-induced renal injury, as measured by both proteinuria and kidney vascular injury score. The extent of L-NAME/AngII-induced renal injury in OVX/E2 rats was comparable to that in the sham-operated, L-NAME/AngII-treated rats.

Within the L-NAME/AngII-treated groups, OVX rats had significantly lower levels of AT_1R protein in the renal cortex than both the sham-operated and OVX/E2 groups (Figure 3b and c). Furthermore, in all groups of L-NAME/AngII-treated rats, the level of AT_1R protein correlated positively with urinary protein excretion on day 14 ($r^2=0.52$, $P<0.001$) (Figure 3d) and with the renal vascular injury score ($r^2=0.24$, $P<0.01$).

Effect of estrogen status on relationship between renal injury, lipid levels, AT_1R protein, and mRNA levels of $AT_{1A}R$ and $AT_{1B}R$

A second set of studies was performed in order to determine, in the same animals, the effects of our treatments on renal cortical levels of AT_1R protein, $AT_{1A}R$ mRNA and $AT_{1B}R$ mRNA, and on hyperlipidemia, a potential mediator of the adverse effects of E2.[16–18] We studied OVX rats receiving one of three treatments: (1) placebo (control treatment); (2) L-NAME/AngII; and (3) L-NAME/AngII plus E2. All rats received drinking water containing 1% NaCl. Rats were fed a phytoestrogen-free diet as phytoestrogens have been shown to mimic the effects of estrogen[19] and we wanted to minimize factors that could potentially confound our results. OVX rats not receiving E2 had lower serum E2 levels, weighed more, and had uterine atrophy compared with those receiving E2 (Table 2). As observed in the first study, E2 treatment did not influence significantly the L-NAME/AngII-induced rise in systolic BP, but significantly increased proteinuria in

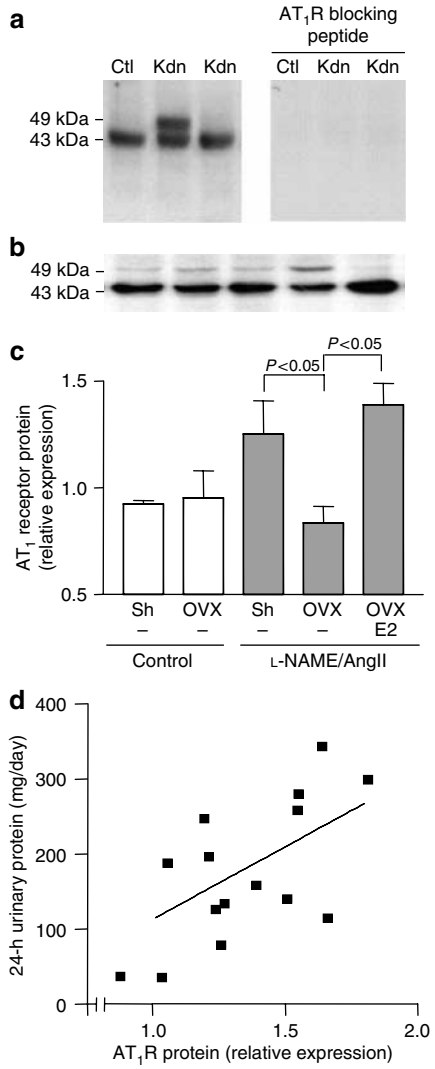

Figure 3 | Effect of L-NAME/AngII and E2 treatments on AT_1R protein content in renal cortex. (**a**) Western blot of rat renal cortical tissue (Kdn) (40 μg/lane) probed with the rabbit monoclonal AT_1R antibody N-10 (sc-1173P). The three lanes on the right were incubated with the AT_1R-blocking peptide before addition of the AT_1R antibody N-10. A positive control for AT_1R protein (Ctl) (12.5 μg/lane of PC-12 lysate (sc-2250, Santa Cruz)) was run in the first and fourth lanes. The AT_1R antibody detected in rat renal cortex two bands with molecular weights of approximately 43 and 49 kDa. The relative intensities of these two bands varied between animals within the same experimental condition. These bands are thought to represent different extents of glycosylation.[38] Neither band was observed when the AT_1R-blocking peptide was used, showing the specificity of the N-10 antibody for AT_1R. AT_1R-blocking peptide did not block detection of ERα (data not shown). (**b**) Representative Western blot of renal cortical tissue (40 μg of protein per lane) showing 43 and 49 kDa bands for AT_1R in the different study groups. Lanes 1–2, control treatment; lanes 3–5, L-NAME/AngII treatment; lanes 1 and 3, sham-operated rats; lanes 2, 4–5, OVX rats. (**c**) Relative levels of AT_1R protein in renal cortex of the different study groups. $n=5$ in sham/control, $n=7$ in OVX/control and in L-NAME/AngII-treated rats, $n=8$ sham, $n=8$ OVX, $n=6$ OVX/E2. Data given as mean $\pm$ s.e. (**d**) AT_1R protein content in renal cortex plotted vs 24-h urinary protein for individual rats in sham ($n=8$), OVX ($n=6$), and OVX/E2 ($n=6$) groups receiving L-NAME/AngII. AT_1R content correlates with proteinuria, $r^2=0.52$; $P<0.001$. Linear regression line is shown.

Table 2 | Effect of L-NAME/AngII and E2 treatments on metabolic measures in OVX rats.

	Control	L-NAME/AngII	
	OVX (n=8)	OVX (n=8)	OVX/E2 (n=8)
Systolic blood pressure (mm Hg)	111 $\pm$ 5	153 $\pm$ 7[a]	158 $\pm$ 5[a]
Uterine weight/body weight (mg/g)	3.1 $\pm$ 0.06	3.3 $\pm$ 0.04	27.2 $\pm$ 3[a,b]
Body weight (g)	324 $\pm$ 7	308 $\pm$ 3	251 $\pm$ 8[a,b]
Serum measurements			
Estradiol (pg/ml)	9.6 $\pm$ 1.3	11.2 $\pm$ 1	191 $\pm$ 120[a,c]
Creatinine (mg/dl)	0.36 $\pm$ 0.1	0.34 $\pm$ 0.1	0.35 $\pm$ 0.1
Lipid profile			
Triglyceride (mg/dl)	160 $\pm$ 17	133 $\pm$ 22	175 $\pm$ 35
Cholesterol (mg/dl)	122 $\pm$ 9	117 $\pm$ 4	122 $\pm$ 7
HDL (mg/dl)	48 $\pm$ 3	45 $\pm$ 1	42 $\pm$ 2
LDL (mg/dl)	15 $\pm$ 1	15 $\pm$ 1	17 $\pm$ 1
24 h urine measurements			
Total protein (mg/day)	5.1 $\pm$ 0.6	5.8 $\pm$ 1.3	21.5 $\pm$ 9.6[a,c]
Creatinine (mg/day)	11.4 $\pm$ 2	16.4 $\pm$ 3	12.2 $\pm$ 2
Creatinine clearance (ml/min)	0.8 $\pm$ 0.1	0.99 $\pm$ 0.2	0.69 $\pm$ 0.1

AngII, angiotensin II; E2, 17β-estradiol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; L-NAME, N^{ω}-nitro-L-arginine-methyl-ester; OVX, ovariectomized.
[a] $P<0.01$ vs OVX group receiving control treatment.
[b] $P<0.01$ vs OVX group receiving L-NAME/AngII treatment.
[c] $P<0.05$ vs OVX group receiving L-NAME/AngII treatment.
Data given as mean $\pm$ s.e.

L-NAME/AngII-treated rats (Table 2). Creatinine clearance was similar in all three groups. E2 treatment did not alter levels of total cholesterol, high-density lipoprotein, low-density lipoprotein, and triglyceride.

Renal cortical levels of AT_1R protein were determined by Western blot analysis using two distinct antibodies, a mouse monoclonal antibody ab9391 from Abcam Inc. (Cambridge, MA, USA) and a rabbit polyclonal antibody sc-1173 from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA), with similar results (Figure 4a and b). Renal cortical levels of AT_1R protein were significantly higher in the E2/L-NAME/AngII-treated OVX rats compared with OVX rats receiving L-NAME/AngII and with OVX rats receiving control treatment.

We examined mRNA expression in renal cortex of $AT_{1A}R$ and $AT_{1B}R$, the two subtypes of AT_1R in rodents. The levels of $AT_{1A}R$ mRNA in L-NAME/AngII-treated OVX rats receiving E2 were significantly elevated compared with those in control-treated OVX rats and L-NAME/AngII-treated OVX rats not receiving E2 (Figure 4c). The level of $AT_{1B}R$ mRNA was highest in the OVX rats receiving E2/L-NAME/AngII and was significantly greater than that observed in estrogen-deficient, OVX rats receiving control treatment (Figure 4d). The level of AT_1R protein assessed with the mouse monoclonoal antibody correlated with the amount of $AT_{1A}R$ mRNA ($r^2=0.58$, $P<0.001$) and with the extent of proteinuria ($r^2=0.40$, $P<0.03$).

Expression of estrogen receptor-α, estrogen receptor-β, and AT_1R in female rat kidney cortex

Confocal microscopy showed expression of estrogen receptor (ER)-α, ER-β, and AT_1R in the arterioles and glomeruli of the

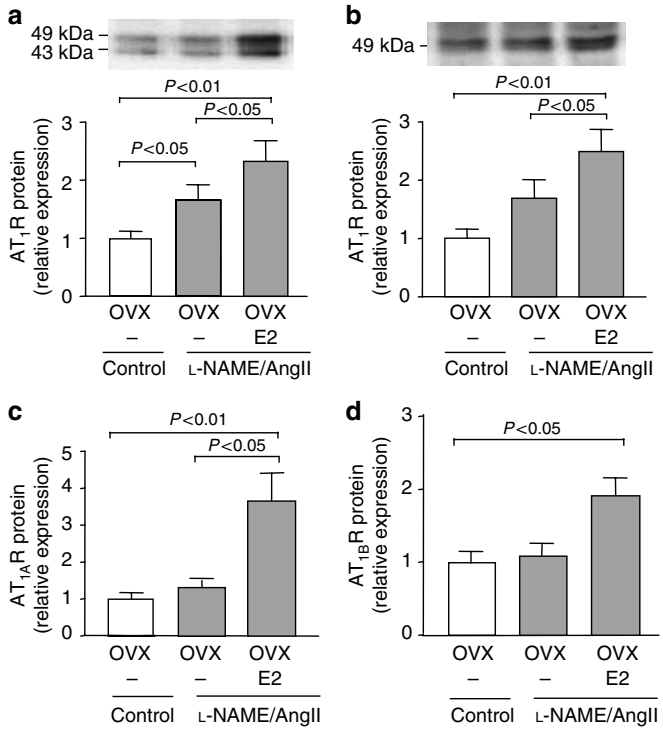

Figure 4 | Effect of E2 on renal cortical levels of AT_1R protein, $AT_{1A}R$ mRNA, and $AT_{1B}R$ mRNA in L-NAME/AngII-treated OVX rats. (**a**) Representative Western blot probed with mouse monoclonal antibody ab9391 and graph quantifying AT_1R protein in the three treatment groups – OVX rats receiving control treatment, OVX rats receiving L-NAME/AngII, and OVX rats receiving E2 plus L-NAME/AngII. (**b**) Representative Western blot probed with rabbit polyclonal antibody sc-1173 and graph quantifying AT_1R protein in the three treatment groups showing results similar to those with the antibody ab9391. (**c**) $AT_{1A}R$ mRNA and (**d**) $AT_{1B}R$ mRNA levels in the three groups. mRNA levels are expressed relative to β-actin mRNA levels. $N = 8$ per group. Data given as mean $\pm$ s.e.

renal cortex of sham-operated, control-treated rats (Figure 5). Merged images showed colocalization of both types of estrogen receptors with the AT_1R. In addition, AT_1R immunoprecipitates from renal cortex homogenates were analyzed by Western blot using antibodies specific to ER-α and ER-β. Figure 6b shows that immunoprecipitation of AT_1R co-precipitates ER-α and ER-β. These studies suggest a close association of the AT_1R with both estrogen receptors.

DISCUSSION

Under conditions of high AngII and low NO, female rats developed hypertension and kidney damage characterized by proteinuria, glomerular injury, and renal vascular injury. Ovariectomy reduced this renal damage, whereas the administration of E2 to OVX rats increased damage. Estrogen-replete, L-NAME/AngII-treated animals had increased renal cortical expression of AT_1R protein compared with estrogen-deficient OVX rats receiving L-NAME/AngII. Furthermore, the amount of AT_1R protein correlated with the extent of L-NAME/AngII-induced proteinuria and renovascular injury. These results suggest that under conditions of low NO availability and high AngII, E2 increased the expression of AT_1R, leading to increased AngII-mediated renal injury. Estrogen-mediated increases in expression of AT_1R may represent a novel mechanism by which E2 increases renal injury and could have widespread implications, given the critical role of AngII in mediating cardiovascular injury in general.

Studies in OVX rats examining the effects of estrogen treatment on renal function have demonstrated both beneficial and adverse effects. These differing results may be related to differences between rat strains and experimental models. Estrogen reduced proteinuria and/or glomerulosclerosis in the OVX rat remnant kidney model,[20] the OVX rat with streptozotocin-induced diabetes,[21] and the OVX, aging Dahl salt sensitive rat fed a low sodium diet.[22] In addition to the present model, administration of estrogen to OVX female rats increased proteinuria and glomerular injury in: (1) the obese Zucker rat;[18] (2) the uninephrectomized hyperlipidemic analbuminemic rat;[16] (3) the spontaneously hypercholesterolemic female Imai rat;[17] and (4) in some,[23] but not all[24] studies the stroke-prone spontaneously hypertensive rat.

In some models of renal injury, estrogen-induced increases in hyperlipidemia are hypothesized to contribute to the increase in renal injury observed with estrogen treatment.[16–18] In the current studies, E2 treatment did not alter lipid levels in L-NAME/AngII-treated rodents, suggesting that the increase in renal injury with E2 was not mediated by changes in lipids. In addition, estrogen status did not influence the effect of L-NAME/AngII on systolic BP, suggesting that the adverse effects of estrogen on proteinuria were not mediated by changes in systemic BP.

In L-NAME/AngII-treated rats, levels of AT_1R protein in the renal cortex were reduced by ovariectomy and increased by E2 treatment. E2 also increased renal cortical levels of $AT_{1A}R$ mRNA in OVX rats treated with L-NAME/AngII. Furthermore, renal cortical levels of AT_1R protein correlated with both proteinuria and the renovascular injury score. Thus, in this AngII-dependent model of renal injury, E2 appeared to increase renal injury by increasing the expression of AT_1R. Similarly, estrogen has been reported to increase AT_1R protein in renal medullary rays[25] and in uteri of healthy OVX rats.[26] In contrast, estrogen has been reported to decrease AT_1R protein in other tissues such as rat adrenal,[26,27] vascular smooth muscle,[28] pituitary[26,29] and myocardium.[30] Thus, the effect of estrogen on expression of AT_1R protein may be tissue specific and/or depend on other factors such as NO and AngII.

These studies do not allow us to determine whether the effect of E2 on AT_1R expression is direct or indirect. It is well known that E2 has effects on components of the renin–angiotensin–aldosterone system. E2 increases expression of angiotensinogen.[31] Thus, the E2 effects on AT_1R could be indirect and secondary to E2-mediated increases in angiotensinogen, leading to increased AngII. However, if this were to occur, we would expect a compensatory decrease in AT_1R.

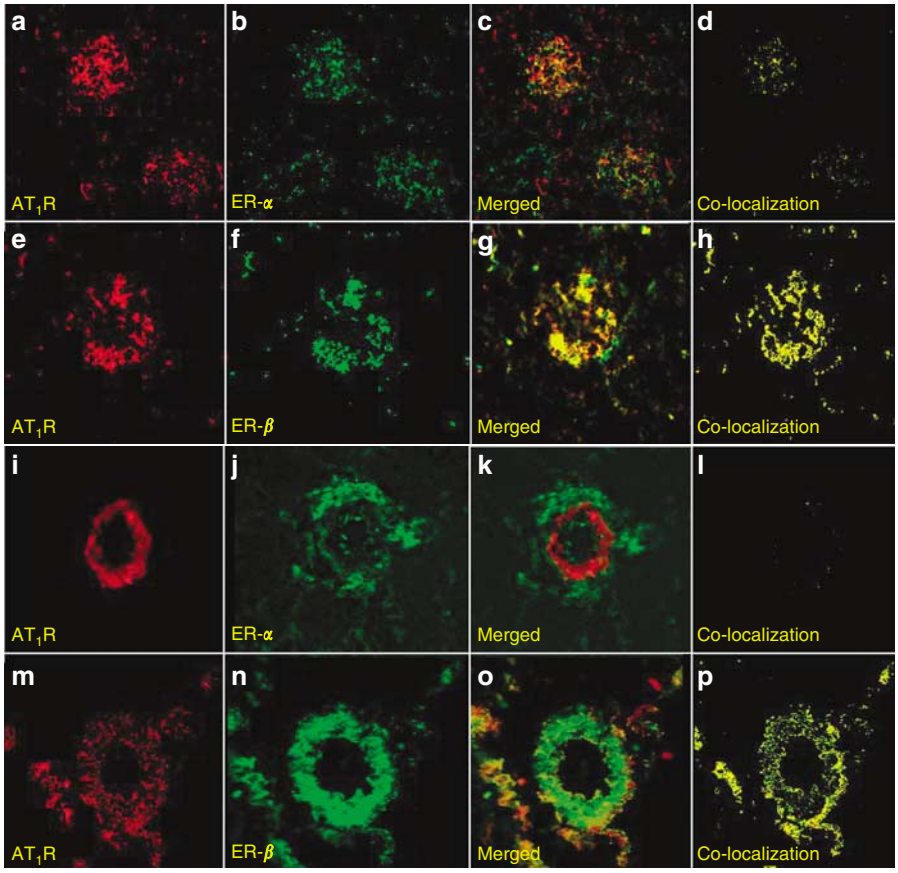

Figure 5 | Confocal microscopy of AT_1R, ER-α, and ER-β in renal cortex. Renal cortical tissue sections from female rats drinking 1% NaCl for 14 days were co-immunostained with fluorescent antibodies to (**a** and **i**) AT_1R and (**b** and **j**) ER-α and to (**e** and **m**) AT_1R and (**f** and **n**) ER-β. AT_1R, ER-α, and ER-β are expressed in (**a–d** and **e–h**) glomeruli and (**i–l** and **m–p**) renal vessels. In glomeruli, AT_1R colocalized with both (**c** and **d**) ER-α and (**g** and **h**) ER-β. In renal arterioles, AT_1R colocalized (**o** and **p**) with ER-β but showed little colocalization (**k** and **l**) with ER-α.

Instead, we saw an increase. To begin to address the possibility that there could be a direct effect of E2 on AT_1R, we used confocal microscopy and immunoprecipitation studies to determine if the estrogen receptors and AT_1R are expressed in the same cells. Because these studies were positive, this suggests E2 may have a direct effect on AT_1R expression. Further studies are needed to address this possibility.

Several studies show an adverse effect of estrogen-containing oral contraceptives and estrogen–progestin replacement therapy on renal vascular injury in women. A study involving over 100 women revealed an increased risk of microalbuminuria in women who used estrogen–progestin oral contraceptives compared with those who did not use oral contraceptives.[11] Similarly, a case–control study of over 4000 women showed that, after adjusting for age, hypertension, diabetes, obesity, hyperlipidemia, and smoking, the risk of microalbuminuria was increased in pre- and post-menopausal women who used estrogen–progestin therapy and that the risk increased with the duration of hormone replacement therapy in postmenopausal women who used hormone replacement therapy.[12] In healthy, premenopausal women, use of estrogen-containing oral contraceptives for at least 3 months was associated with an increase in both renal vascular resistance and filtration fraction as compared with values in women not using oral contraceptives.[32] Finally, a recent study examined the effect of oral contraceptive use on renal blood flow and activity of the renin–angiotensin system in premenopausal women with and without diabetes.[13] The oral contraceptive users, as compared to non-users, had a greater increase in renal blood flow with acute administration of an angiotensin-converting enzyme inhibitor suggesting that there is increased activity of the renin–angiotensin system in the renovasculature of women with and without diabetes who use oral contraceptives. This result is consistent with our current finding of increased renal expression of AT_1R protein and mRNA with E2 treatment of rats receiving L-NAME/AngII.

There are several limitations to the current studies. First, E2 treatment was started 7–10 days after ovariectomy. It is possible there were changes in gene expression during this period of estrogen deficiency that were not reversed by initiation of estrogen replacement. Second, we administered E2 continuously achieving average E2 levels of 140–190 pg/ml, whereas E2 levels fluctuate in cycling female rats peaking at 50–150 pg/ml in proestrus (reviewed by Becker *et al.*[33]).

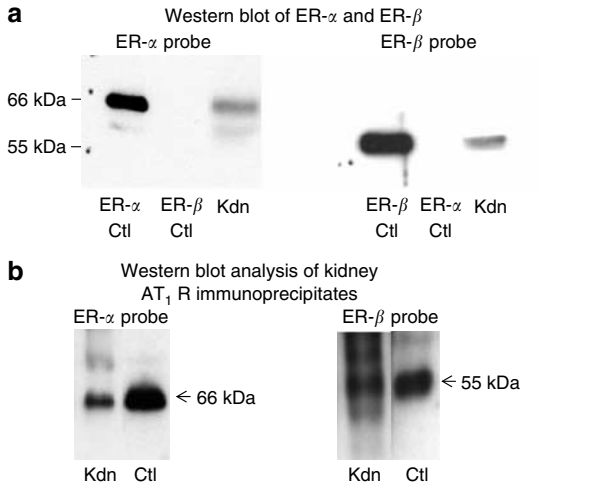

Figure 6 | Expression of AT_1R, ER-α, and ER-β in renal cortex. (**a**) Western blot of rat renal cortical tissue (Kdn) (40 μg/lane), recombinant ER-α (ER-α Ctl), and recombinant ER-β (ER-β Ctl) probed with antibodies specific to ER-α (left panel) and ER-β (right panel). The ER-α antibody detected a 66-kDa band in rat kidney that corresponded to the size of the recombinant ER-α control. The ER-β antibody detected a 55-kDa band in rat kidney that corresponded to the size of the recombinant ER-β control. There was no cross reactivity. (**b**) Representative Western blot analysis of AT_1R immunoprecipitates of kidney cortical homogenates (Kdn) probed for ER-α (left panel) and ER-β (right panel) showing co-immunoprecipitation of AT_1R with both ER-α and ER-β. Recombinant ER-α and ER-β proteins were run as positive controls (Ctl) in right lane of the corresponding blot.

It is possible that, in L-NAME/AngII-treated rats, continuous levels of E2 in the high physiological range could have different effects on renovascular injury than transient exposures to similar levels of E2 in cycling rats. However, cycling female rats are exposed repeatedly, every 4–5 days, to the high E2 levels observed in proestrus. In addition, some studies report prolonged physiological effects of transient increases in circulating E2 (reviewed by Becker *et al.*[33]). Thus, although the current studies demonstrated an adverse renovascular effect of E2 in the 2-week L-NAME/AngII model, longer time periods may be necessary to demonstrate an effect, or the effect may not occur, under conditions of cycling E2 levels or lower continuous doses of E2. Third, we have not examined the effects of different modes of E2 administration on our endpoints. Fourth, although the effects of OVX and E2 treatment are of similar magnitude on renal injury and AT_1R expression in L-NAME/AngII-treated rats, these studies do not rule out the possibility that E2 has effects that are independent of those of OVX. For example, ovariectomy results in progesterone deficiency, and we have not examined the effects of progesterone on renal injury in this model.

By manipulating the estrogenic status of female rats through ovariectomy and estradiol replacement, we showed that the estrogen-replete state was associated with increased renal vascular injury and proteinuria in hypertensive, L-NAME/AngII-treated rats consuming a moderately high sodium diet. Increased renal damage appeared to be mediated by increased expression of AT_1R in the renal cortex. Because of the key role of AngII in mediating renal injury in humans, these findings could have important implications for woman's health, particularly for disease states associated with low NO and high AngII levels such as hypertension.

MATERIALS AND METHODS

Experimental animals

Experiments used 10-week-old female Wistar rats (Charles River Lab, Wilmington, MA, USA) that underwent bilateral ovariectomy (OVX) or sham-operation (sham) at 8 weeks of age. Rats had *ad libitum* access to drinking fluid. They were housed in individual metabolic cages in a climate-controlled environment ($22 \pm 1^{\circ}C$) with a 12-h light, 12-h dark cycle. All experimental procedures met guidelines of the Institutional Animal Care and Use Committee at Harvard University.

Experimental procedures

Two animal studies were performed. In study 1, we examined five groups of rats receiving Purina Lab Chow 5001 (Ralston Purina Co., St Louis, MO, USA) and 1% NaCl to drink: (1) sham-operated rats implanted with pellets containing placebo and receiving control treatment (no L-NAME and minipumps containing vehicle), $n = 8$; (2) OVX rats implanted with pellets containing placebo and receiving control treatment, $n = 8$; (3) sham-operated rats implanted with pellets containing placebo and receiving L-NAME/AngII treatment, $n = 10$; (4) OVX rats implanted with placebo pellets and receiving L-NAME/AngII treatment, $n = 10$; and (5) OVX rats implanted with E2 pellets and receiving L-NAME/AngII treatment, $n = 10$. In study 2, we examined three groups of rats receiving a phytoestrogen-free diet (No. D10001, Research Diets Inc., New Brunswick, NJ, USA) and 1% NaCl to drink: (1) OVX rats implanted with pellets containing placebo and receiving control treatment, $n = 8$; (2) OVX rats implanted with placebo pellets and receiving L-NAME/AngII treatment, $n = 8$; and (3) OVX rats implanted with E2 pellets and receiving L-NAME/AngII treatment, $n = 8$.

Pellets containing E2 (0.5 mg/pellet, 21-day release, No. E121, Innovative Research of America, Sarasota, FL, USA) or placebo (No. C111, Innovative Research of America) were implanted subcutaneously in each rat 7–10 days after ovariectomy or sham operation. E2 levels rise to 50–150 pg/ml during proestrus.[33] The 0.5 mg, 21-day E2 pellets raise plasma estradiol levels to this range.[34–36] One week after implantation of the pellets, animals were treated with L-NAME/AngII as previously described.[15,37] Briefly, rats received 1% NaCl in drinking water or 1% NaCl plus L-NAME. L-NAME (Sigma, St Louis, MO, USA) (40 mg/kg/day) was administered for 14 days either through the drinking water (study 1) or via a subcutaneously implanted pellet (Innovative Research of America) (study 2). Vehicle or AngII (Sigma) (0.225 mg/kg/day) was administered via Alzet osmotic minipumps (Model 2001, Durect Corporation, Cupertino, CA, USA) (1.0 μl/h, 7 days) for the final 3 days. On day 14, rats were anesthetized with isoflurane and blood and tissue samples were harvested.

Measurements and assays

Daily food intake, water intake, body weight, and urine output were recorded. Systolic BP was measured in conscious animals by tail-cuff plethysmography (Blood Pressure Analyzer, Model 179, IITC Life

Science, Woodland Hills, CA, USA).[15] Protein was measured in urine with the Coomasie Plus protein assay (Pierce, Rockford, IL, USA). Creatinine was measured using the ACE creatinine reagent (Alfa Wassermann Inc., West Caldwell, NJ, USA). Serum estradiol was measured with the DPC Double Antibody Estradiol radioimmunoassay (DPC Diagnostic Products, Los Angeles, CA, USA). Lipid levels were detected using ACE lipid panel reagents (Alfa Wasserman). The lipid panel consists of an enzymatic and colorimetric assay for total cholesterol (No. SA1010) and for triglycerides (No. SA1023), and a homogeneous enzymatic colorimetric method for high-density lipoprotein (No. SA1015) and low-density lipoprotein (No. SA1018).

Tissue processing and histological evaluation

One kidney per rat was frozen immediately after collection and stored in liquid nitrogen until processing for Western blot analysis and mRNA studies. The other kidney was processed into paraffin blocks. Coronal kidney sections (5 μm) stained with hematoxylin and eosin or periodic acid-Schiff reagent were examined by light microscopy by a pathologist unaware of the treatment group assignment. Renal arterial and arteriolar damage was defined as the presence of fibrinoid necrosis of the vascular wall irrespective of magnitude of damage in a given vessel. Renal vascular injury was assessed by counting the total number of profiles of damaged arteries and arterioles per mid-coronal section, and dividing by the total number of glomeruli in the same section to normalize for the amount of tissue examined. Vascular injury score was expressed as the number of injured vessels per 100 glomeruli.

Western blot analysis

The kidney cortex was dissected on ice and homogenized in ice-cold 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 6.5 (25 mM MES (Sigma), 150 mM NaCl, 1% Triton X-100, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid) containing 1 mM sodium orthovanadate, the protease inhibitor cocktails P8340 (Sigma) and P2714 (Sigma) each 0.3 ml/10 ml homogenate buffer and the phosphatase inhibitor cocktails P-5726 and P-2850 each 0.3 ml/10 ml homogenate buffer. Homogenates were centrifuged at 11 000 r.p.m. for 10 min at 4°C and the supernatants collected on ice. Protein concentration in the supernatant was determined using a Micro bicinchoninic acid protein reagent kit (Pierce). Supernatants (40 μg of protein) were combined with an equal volume of 2 × laemmli-loading buffer (250 mM Tris, pH 6.8, 10% sodium dodecyl sulfate, 50% glycerol, 0.1% pyronin, 200 mM dithiothreitol), boiled for 5 min, and size fractionated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked in 5% non-fat dried milk in phosphate-buffered saline (PBS) (150 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 anhydride, and 0.2% Triton X-100), and incubated overnight at 4°C with 1:500 AT_1R antibody (rabbit polyclonal antibody N-10; sc-1173, Santa Cruz Biotechnology Inc. or mouse monoclonal antibody ab9391 (Abcam Inc.) to detect AT_1R. We demonstrated excellent correlation between levels of AT_1R protein in rat tissue as determined by radioligand binding with AngII-labeled ((125I)(Sar1, Ile8) and Western blot analysis using either AT_1R antibody, ab9391 ($R = 0.7$, $P < 0.0001$), and sc-1173 ($R = 0.47$, $P < 0.0001$). In addition, for the sc-1173 antibody, additional membranes were incubated overnight with 100 μg of the AT_1R-blocking peptide (N-10P, No. sc-1173P, Santa Cruz Biotechnology Inc.). Sc-2250 (Santa Cruz Biotechnology Inc.) was used as a positive control for AT_1R protein. Equal loading was assessed by reprobing membranes with an antibody to β-actin (1:20 000; Clone AC-15, Sigma). After overnight incubation, the bound antibody was detected by enhanced chemiluminescence (Western Lightning Reagent Plus, Perkin-Elmer Life Sciences, Boston, MA, USA) with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (No. A-6154, Sigma). Gels were scanned using the Epson Perfection 1650 scanner and densitometric analysis was performed with the Imagequant 5.2 software (Molecular Dynamics, Piscataway, NJ, USA). All values were expressed relative to the average value of the OVX rats receiving control treatment, arbitrarily assigned a value of 1.0.

Western blot analysis to detect ER-α and ER-β was performed using mouse monoclonal antibody to ER-α (ERα-1000, No. SRA-1000, Stressgen, Victoria, BC, Canada) that recognizes the activated 4S ER-α and not the inactive 8S oligomeric state,[38] and rabbit affinity purified immunoglobulin G antibody to ER-β (No. PA1-310B, Affinity Bioreagents, Golden, CO, USA), respectively. Recombinant ER-α (No. 330655, Calbiochem, La Jolla, CA, USA) and ER-β proteins (No. 330657, Calbiochem) were run as positive controls.

Immunoprecipitation of AT_1R, ER-α, and ER-β proteins

Magnetic immunoprecipitation was performed using the μMACS system as described by the manufacturer (Miltenyi Biotec, Auburn, CA, USA). Briefly, kidney cortex was homogenized in radioimmunoprecipitation assay buffer pH 8.0 (150 mM NaCl, 50 mM Tris/HCl, 10 mM NaF, 5 mM ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, and 1% Nonidet P-40) supplemented with leupeptin 10 μg/ml, pepstatin A 10 μg/ml, 0.5% deoxycholic acid, aprotinin 20 μg/ml, protease and phosphatase inhibitor cocktails (P-5726, P-2850, P-8340, P-2714; Sigma), and 1 mM sodium orthovanadate. The homogenate (500 μg protein) was incubated for 1 h on ice with 5 μg of antibody to AT_1R (rabbit polyclonal antibody N-10; No. sc-1173, Santa Cruz Biotechnology Inc.) and 100 μl of protein A-Sepharose magnetic microbeads followed by application to a microcolumn. The microcolumn was placed in the field of a magnetic separator, washed sequentially with high and low salt washes and the samples eluted with 1 × boiling laemmli sample buffer. Eluants were probed for ER-α and ER-β by Western blot.

Double labeling of renal cortex for AT_1R, ER-α, and ER-β for confocal microscopy

Confocal microcopy was performed using tissue snap frozen in isopentane. Cryosections (10–12 μm) of kidney tissue were air-dried and fixed with −20°C acetone. After a 10-min exposure to 0.5% Triton X-100 dissolved in PBS, tissue slices were covered for 30 min with a 0.1 M glycine solution, followed by 10 min with blocking solution (1% bovine serum albumin in PBS) containing 1% normal, preimmune serum. After blocking, the tissue slices were incubated at 4°C overnight with the following primary antibodies: rabbit polyclonal AT_1R N-10 antibody (sc-1173, Santa Cruz Biotechnology Inc.), mouse monoclonal AT_1R antibody (TONI-1, No. ab9391-1, Abcam), mouse monoclonal antibody to ER-α (ER-α-1000, No. SRA-1000, Stressgen), and rabbit affinity-purified immunoglobulin G antibody to ER-β (No. PA1-310B, Affinity Bioreagents).

The tissue slices were then washed three times with 0.5% blocking solution in PBS, and incubated for 30 min at 37°C with a secondary antibody or a mixture of antibodies tagged with Alexa

488 and/or Alexa 568. After the final incubation, the tissue slices were washed sequentially with PBS and deionized water. The air-dried slices were mounted with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and kept in a dark cool place.

Confocal microscopy

Sequential images were obtained with a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany). The fluorophors Alexa 488 and Alexa 568 were excited with the 488 and 543-nm laser lines, respectively, and the signals collected with a BP filter 505–530 nm for Alexa 488 and 560–615 nm for Alexa 568. The autofluorescence of the samples was minimal at the excitation wavelengths and was subtracted from the values obtained during measurements. When antibody-blocking peptides were not available, fluorescence produced without the addition of the primary antibody was subtracted from that obtained with the primary antibody.

Analysis of mRNA expression by real-time polymerase chain reaction

Total mRNA was extracted from kidney cortex using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's protocol. cDNA was synthesized from 1.5 μg RNA with the First Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, UK). Polymerase chain reaction amplification reactions were performed in duplicates by ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the QuantiTect SYBR Green PCR kit (Qiagen Sciences) according to the manufacturer's protocol. Primers were designed with Primer Express version 2.0 (Applied Biosystems) and were synthesized by Sigma-Genosys (Woodlands, TX, USA). The sequences of the primers used to detect specific genes were as follows: $AT_{1A}R$: 5′-ACCAGGTCAAGTGGATTTCG-3′ (forward, nucleotide 208), 5′-ATCACCACCAAGCTGTTTCC-3′ (reverse, nucleotide 417); $AT_{1B}R$: 5′-ACTGCACACGGTGCATTTTA-3′ (forward, nucleotide 97), 5′-ATCACCACCAAGCTGTTTCC-3′ (reverse, nucleotide 306). The relative level of mRNA expression of a specific gene was calculated based on $\Delta\Delta$CT method according to the manufacturer's instruction and normalized to mRNA level for the housekeeping gene β-actin. The results were confirmed with Taqman Assay-on-Demand method (Applied Biosystems).

Statistical analysis

The statistical significance of the differences between group means for the data was determined by one-way analysis of variance followed by Newman–Keuls *post hoc* test for multiple comparisons. Correlations between different parameters were analyzed by simple linear regression. Differences in means with P-values $\leqslant 0.05$ were considered statistically significant. Values are expressed as mean $\pm$ s.e.

ACKNOWLEDGMENTS

This work was supported by NIH training grants 5T32HL007609 and DK07529 (CG), and NIH research grants HL-63423 and HL-07718. This work was presented in part at the 19th Scientific Meeting of the International Society of Hypertension, 2003, Prague, Czech Republic and at the American Heart Association, Scientific Sessions 2004, New Orleans, Louisiana.

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